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Physiological approach to maturation of brown adipocytes in primary cell culture

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Abstract

Molecular and metabolic aspects of differentiation of brown adipocytes of the Djungarian hamster (*Phodopus sungorus*) were studied in primary culture. Expression of uncoupling protein and lipoprotein lipase were investigated by Western and Northern blotting and indirect immuno-fluorescence microscopy. The activity of 5′-deiodinase type II was determined by a radioactive enzyme assay. Activity of cytochrome-*c*-oxidase and cell respiration rates were measured with a Clark electrode. We evaluated functional differences of developmental stages by measuring the reaction to β-adrenergic stimulation throughout the differentiation process. The results show that differentiation of hamster brown adipocytes is an at least two-step development with physiologically discriminable cell types. Generation of triiodothyronine (T3) from thyroxine by activation of the 5′deiodinase occurs in immature brown adipocytes and is mediated primarily by β1- rather than β3-adrenergic receptors. The thermogenic capacity is subsequently increased in mature brown adipocytes. β-Adrenergic receptor stimulation increases UCP expression of mature adipocytes but is not able to recruit new brown adipocytes. © 1997 Elsevier Science B.V.

Keywords: Brown adipocyte; Uncoupling protein; 5′-Deiodinase type II; Cell respiration; Functional cell type

1. Introduction

Keeping energy budget in balance belongs to one of the major challenges small mammals face under unfavorable climatic and nutritional conditions. Brown adipose tissue (BAT) plays a key role in energy homeostasis during cold exposure, hibernation, daily torpor, and in the time after birth in many mammal species [1]. Its unique function is the production of heat by uncoupling the respiratory chain from ATP production. Responsible for heat production is the uncoupling protein (UCP), which is localized in the inner mitochondrial membrane. White adipose tissue (WAT) is looked upon as the physiological counterpart of BAT, since its main function lies in energy storage, while BAT is an energy dissipater [1,2]. This antagonistic function is underlined by histological differences and physiological contrary regulation. White fat cells are unilocular cells, while brown fat cells are multilocular. The morphology of white and brown fat mitochondria differs according to their respective metabolic function. Brown fat cells have high density of mitochondria with large membrane surface and high respiratory capacity [3,4]. Although the two fat cell types are easily distinguished by tissue localization, morphology and

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metabolism, it is not yet known, at which point of cellular development brown fat cells start to express their tissue specific physiological features. It is widely accepted that cold induced increase of thermogenic capacity in BAT is dependent on local generation of triiodothyronine (T3). Activation of 5′-deiodinase type II (5′D-II) in BAT by norepinephrine is a permissive step during brown fat cold adaptation [5,6]. In parallel primary cultures of brown and white preadipocytes of the Djungarian hamster we already showed in preliminary studies that T3 specifically increases UCP expression in brown adipocytes [7]. We now present data illuminating the differentiation dependent effect of T3 in brown adipocyte primary culture and suggest a key role of 5′D-II activation by norepinephrine as a permissive step in the differentiation program of brown adipocytes. Furthermore, we propose that a shift in the relative amounts of cell sub populations in BAT might be a possible explanation for how thermogenic capacity is increased, although tissue mass is reduced and cell number not changed during cold acclimation of the Djungarian hamster [8].

2. Materials and methods

Primary cell cultures were obtained from axillary, suprasternal, interscapular, and dorso-cervical brown fat depots (BAT) and inguinal white fat depot (WAT) of Djungarian hamsters. Tissues of 5–10 hamsters at the age of 4–6 weeks (about 1 g BAT and 1 g WAT per animal) were pooled and stromal-vascular fraction was obtained after collagenase treatment and cultured in 10% fetal calf serum (FCS) as described [7,9]. Medium was changed at day 1 and 3. At the third day FCS was reduced to 7% and insulin (17 nM) and triiodothyronine (1 nM) were added. For the harvest of cells medium was aspirated and dishes with cells were frozen at −20°C until further analysis or immediately suspended for measurements of cell respiration.

O_2-consumption of the cells was measured with a Clark electrode as described [7]. Unstimulated respiration was recorded for several minutes followed by the addition of the β-adrenergic agonist isoproterenol (1 μM). We thus obtained O_2-records for unstimulated and stimulated respiration for each cell population.

Total cell homogenates were prepared by sonifying the cells for 10 s in 200 μl PBS. Protein content was determined by the method of the BCA micro assay [10]. A 10 μg sample was used for SDS-PAGE and immuno-blotting. UCP was detected using a polyclonal rabbit antibody against Djungarian hamster UCP as described, using a Djungarian hamster brown fat mitochondrial preparation as a standard [7]. We used Hybond-C membranes (Amersham) for blotting and enhanced chemoluminescence kit (ECL, Amersham) as detection system. Unspecific binding sites were saturated with 20% FCS, 1% casein, and 0.05% Tween 20 in PBS. Signals were quantified by densitometrical scanning of the films.

Northern blot analysis was performed as described [9]. RNA was extracted from cells according to the single step acid phenol-guanidine protocol [11]. Total RNA was separated by electrophoresis in a 1.2% agarose gel containing formaldehyde and blotted by capillary transfer to a nylon membrane (Hybond N, Amersham). Rat UCP-cDNA and bovine LPL-cDNA were labeled with ^{32}P-ATP using a random prime kit (Boehringer) and hybridized with total RNA. Blots were washed at high stringency and then subjected to autoradiography. The relative UCP- and LPL- mRNA levels were quantified densitometrically. Rat UCP and bovine LPL cDNAs were gifts from D. Ricquier, Paris and M. Schotz, Los Angeles, respectively.

Measurement of 5′D-II activity was performed essentially as described [12]. Cells from petri dishes with 10 cm diameter were detached by scraping with a rubber blade into 1 ml of PBS and sedimented by 10 min centrifugation at 14 000 × g at room temperature. They were homogenized by sonification and stored at −80°C for further analysis. Each assay was performed in duplicate. Protein content was determined by the method of BCA-microassay. Activity of 5′D-II is expressed in fmol iodide/h and mg protein.

Indirect immuno-fluorescence microscopy was performed as described [7]. Suspensions of stromal-vascular fraction of brown or white fat tissues were dropped into wells of a multiwell-coverslip. After 2 h samples were covered with medium and cells were cultured as indicated above. For the calculation of the relative amount of UCP and LPL per adipocyte we used a Zeiss photomanager connected to the microscope. Reciprocal values of automatic spot exposure times for small homogeneous cell groups (4–6 cells)

were registered. For each value we measured at least 10 groups of cells. All cells that were compared with each other had been treated simultaneously and identically. Therefore we considered the intensity of fluorescence to be directly proportional to the amount of intracellular protein. Although this seems to be a rather rough method, results were homogeneous and correlated very well with results of Western and Northern blots.

Statistical analysis was performed using paired-t-test and analysis of variance followed by Duncan's test. The level of significance was set at $P < 0.05$. Values are given as mean ± S.E.M.

3. Results

3.1. Morphological and cytological aspects of differentiation

Brown adipocyte primary culture starts with only few preadipocytes from the stromal vascular fraction of dispersed brown adipose tissue. From the first to the 8th day cell number increased 7-fold from 169 cells/mm^2 to 1177 cells/mm^2 (Fig. 1). Confluence was reached at day 5 and adipogenesis started at day 6 of culture. At the 8th day 84% of the cells were morphologically categorized as adipocytes. From day 8 to 9 there was a significant loss of adipocytes (444 cells/mm^2). Maturating adipocytes were able to grow in up to four layers. We defined day 1–8 as the period of proliferation and day 6–10 as the period of differentiation. Since our main interest was focused on the developmental stages of maturating adipocytes in culture, we restricted our investigations to the period of differentiation.

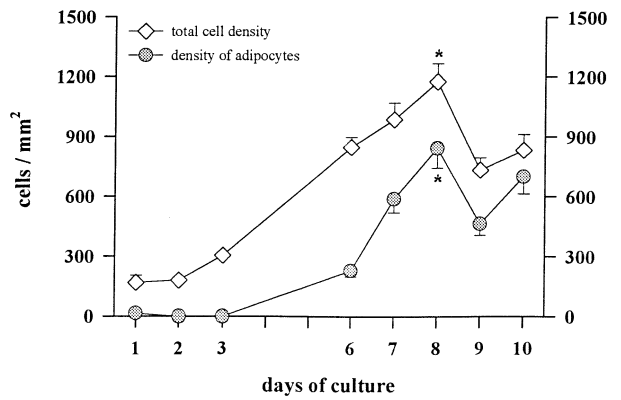

Fig. 1. Proliferation and adipose conversion of brown fat cell primary culture. Total cell density was determined by counting DAPI-stained nuclei per mm^2. Cells were categorized as adipocytes, when they were of rounded shape including multilocular fat droplets. Shown are means ± S.E.M. Asterisks * indicate values significantly different from days 1 to 7 and 9. ($P < 0.05$, ANOVA, DUNCAN, $n = 11–38$)

By double-labeling cells both for LPL and UCP with FITC- and TRITC-coupled secondary antibodies respectively, we were able to distinguish immature and mature brown adipocytes. LPL is an early marker of adipogenesis, while UCP is considered to be a late gene of differentiation in brown adipocytes, at least under the described experimental conditions [9,12,13]. LPL could never be detected in fibroblast like preadipocytes. Simultaneously with the onset of morphological change into the typical rounded fat cell shape, LPL was detectable. Under the described culture conditions UCP was expressed exclusively in cells that were morphologically categorized as adipocytes. UCP expression occurred one day later than LPL. Therefore we considered adipocytes that were positively stained for LPL but negative for UCP as immature and adipocytes that were positively stained for both proteins as mature (see [7]).

This made it possible to distinguish between effects of β-adrenergic stimulation on recruitment of UCP expressing cells out of the pool of immature adipocytes (lacking UCP) and effects on thermogenic capacity of mature adipocytes. Stimulation of the cells with 0.1 μM isoproterenol for 24 h lead to recruitment of UCP expressing cells only between the 7th and 8th day of culture. Density of UCP expressing cells increased by 244 cells/mm^2, which corresponds to a 30% increase (not shown). At no other day such a recruitment was observed.

3.2. Metabolic aspects of differentiation

Metabolic rate of cultured cells was determined with a Clark electrode using suspended cells. O_2-consumption of brown fat cell populations increased significantly from 13.1 at the 6th day to 47.4 nmol O/min/10^6 cells at the 10th day (Fig. 2). White adipocytes of the same animals were cultivated in parallel as a control. White adipocytes showed no significant change in metabolic rate during the time

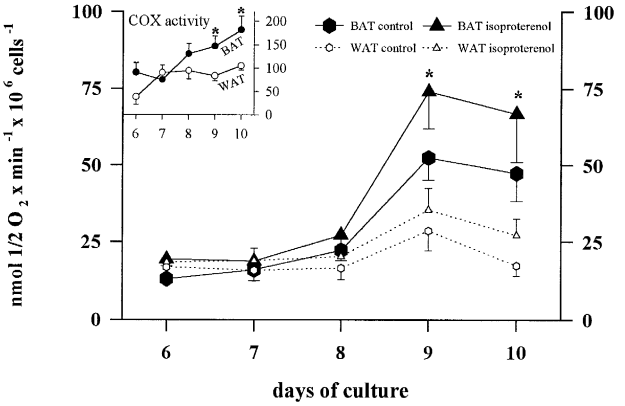

Fig. 2. Respiration rates of unstimulated and stimulated brown (filled symbols) and white (open symbols) adipocytes in primary culture. Cells were acutely stimulated with 1.0 μM isoproterenol in the reaction chamber of the clark electrode. Oxygen consumption was measured in suspensions of intact cells. Inset: Respiratory capacity of brown and white adipocytes was determined by measurements of the activity of cytochrome-*c*-oxidase in cell homogenates. Shown are mean $\pm$ S.E.M. Asterisks * indicate values significantly different from the corresponding values for white adipocytes. ($P < 0.05$, ANOVA, DUNCAN, $n = 3$–12).

course of differentiation (19.8 nmol O/min/10^6 cells). The difference between white and brown adipocytes in regard to oxygen consumption was significant at the 9th and the 10th day of culture (Fig. 2). Maximal O_2-consumption was measured in brown adipocytes at the 9th day (52.4 nmol O/min/10^6 cells).

The metabolic reaction of the cells to β-adrenergic stimulation was examined by adding 1 μM isoproterenol into the chamber of the Clark electrode during the measurement of cellular O_2-consumption. This reaction of adipocytes to acute β-adrenergic stimulation was also determined in parallel cultures of brown and white adipocytes. At any stage of differentiation either cell type reacted with a slight but significant increase of metabolic rate after treatment with isoproterenol. Significantly different metabolic reactions of brown and white adipocytes occurred only on days 9 and 10, i.e. at a late stage of differentiation. Maximal respiration rates were observed at day 9 with 74.1 nmol O/min/10^6 cells in brown versus 35.4 nmol O/min/10^6 cells in white adipocytes (Fig. 2).

To determine the respiratory capacity of the cells, the activity of cytochrome-*c*-oxidase (COX-activity) was measured in cell homogenates. From the 6th to the 10th day respiratory capacity doubled from 91.6 to 182.2 nmol O/min/10^6 cells in brown adipocytes. At the 9th and the 10th day of culture COX-activity of brown adipocytes was significantly elevated in comparison to white adipocytes (Fig. 2, inset).

Taken together, differentiating brown adipocytes showed tissue specific metabolic properties and behavior only at a late stage of differentiation (day 9 and 10). During the early stage (day 6–8) metabolic features of white and brown adipocytes were the same, although the two cell types could be discriminated morphologically [7].

3.3. Molecular aspects of differentiation

For determination of differentiation at the molecular level we examined the time course of expression of lipoprotein lipase (LPL), uncoupling protein (UCP), and of the activity of the 5′deiodinase type II (5′D-II). LPL served merely as a marker for early differentiation, while UCP served as a marker for late differentiation as well as tissue specific marker. Activity of 5′D-II is supposed to play a permissive role for the expression of UCP during cold acclimation in vivo [5,6].

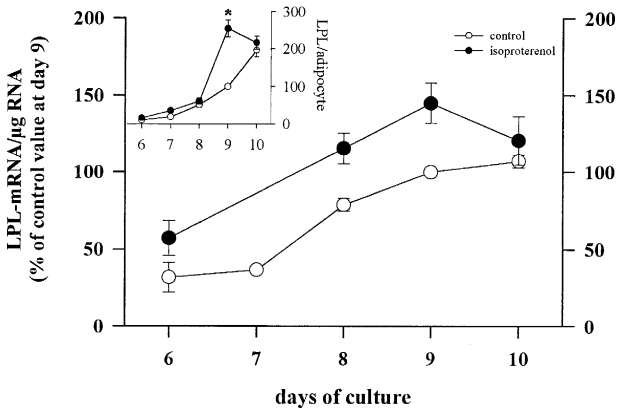

Fig. 3. LPL expression in primary cultures of brown adipocytes was determined by Northern blotting of total RNA. Shown are mean values $\pm$ S.E.M. of unstimulated (open symbols) and stimulated (filled symbols) primary cultures. Cells were stimulated by 0.1 μM isoproterenol for 24 h. Control values of day 9 of culture were set to 100% ($n = 2$–9). Inset: Fluorescence intensity of FITC-labelled LPL in unstimulated (open symbols) and stimulated (filled symbols) brown adipocytes. Asterisks * indicate values significantly different from the corresponding control value. ($P < 0.05$, ANOVA, DUNCAN, $n = 10$–21).

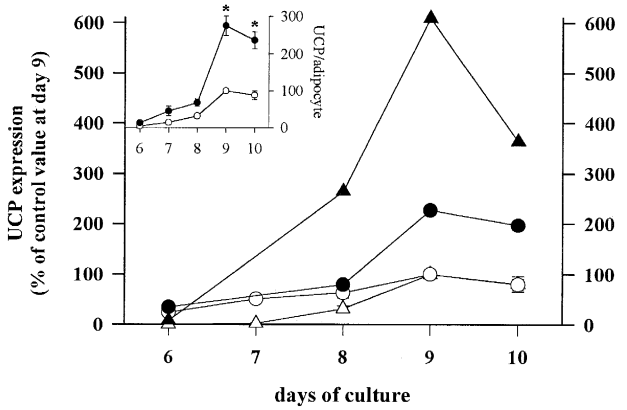

Fig. 4. UCP expression in primary cultures of brown adipocytes was determined by Northern and Western blots. Shown are mean values ± S.E.M. of unstimulated (open symbols) and stimulated (filled symbols) UCP expression at the transcriptional level (UCP-mRNA/μg RNA: triangles) and at the translational level (UCP/mg protein: circles). Cells were stimulated by 0.1 μM isoproterenol for 24 h. Control values of day 9 of culture were set to 100% ($n = 2–13$). Inset: Fluorescence intensity of TRITC-labelled UCP in unstimulated (open symbols) and stimulated (filled symbols) brown adipocytes. Asterisks * indicate values significantly different from the corresponding control values. ($P < 0.05$, ANOVA, DUNCAN, $n = 10–21$).

3.4. Lipoprotein lipase

LPL-mRNA/μg total RNA increased from the 6th to the 10th day by 30% (Fig. 3), while the cell-bound activity of this enzyme increased by factor 12 (data not shown). This coincides with the measured 10-fold increase of intracellular LPL protein (Fig. 3, inset). β-Adrenergic stimulation of the cells with 0.1 μM isoproterenol for 24 h had negligible effect on LPL-mRNA/μg RNA. Intracellular LPL protein was elevated at the 9th day of culture by 250%. At all other days isoproterenol failed to stimulate LPL synthesis (Fig. 3). The stimulating effect as well as the interaction of time and stimulus (i.e., differentiation dependent reaction) were highly significant.

3.5. Uncoupling protein

The expression of UCP was detected one day later than the expression of LPL. UCP-mRNA concentration showed a 20-fold increase from day 6 to day 10, while UCP protein only increased 4-fold (Fig. 4). A significant and abrupt increase of mRNA and Protein occurred between day 8 and 9. But the relative amount of UCP expressing cells in culture did not change after day 7, when about 50% of adipocytes were positively stained for UCP (data not shown). Intracellular fluorescence, i.e. UCP expression, intensified by factor 20 from day 6 to 10 with a sudden increase after day 8 (Fig. 4, inset).

After β-adrenergic stimulation a marked increase in UCP concentration could be detected in Northern and Western blots from day 8 to 10. UCP-mRNA increased at day 8 by 200%, at day 9 by 600%, and at day 10 by 400% (Fig. 4). At the translational level there was a delay of about one day: only a slight increase at day 8, and a 200% increase at the days 9 and 10. Fluorescence of TRITC-coupled UCP intensified by 300% after β-adrenergic stimulation at days 9 and 10, but only very little at days 6 to 8 (Fig. 4, inset).

3.6. 5′-deiodinase Type II

Under our experimental conditions the activity of the 5′D-II did not change during the maturation

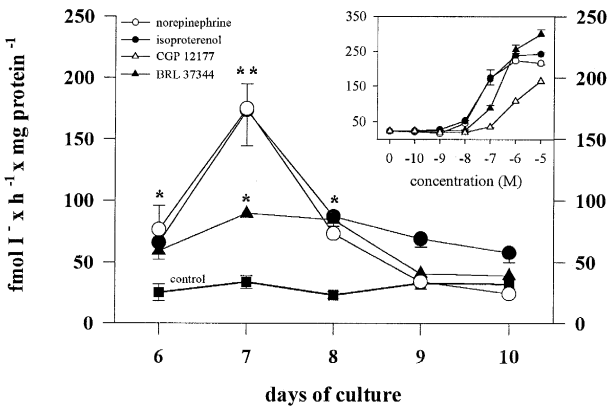

Fig. 5. 5′Deiodinase activity of brown adipocytes in primary culture. Cells were stimulated for 8 h with 0.1 μM isoproterenol (filled circles), norepinephrine (open circles), and BRL 37344 (filled triangles). Asterisks * indicate values significantly different from the corresponding control value, while two asterisks ** indicate values significantly different from all other values ($P < 0.05$, ANOVA, DUNCAN, $n = 4–23$). Inset: Dose response curve for isoproterenol, norepinephrine, BRL 37344, and CGP 12177 (open triangles). Shown are means ± S.E.M. of 4–10 measurements.

process of adipocytes in primary culture (28.2 fmol iodide/mg protein/h, Fig. 5). It is important to emphasize that the culture medium was supplied with 1 nM triiodothyronine (T3) from the third day on. Thus there was no substrate inhibition by thyroxin [14] and no stimulation by product deficiency [15].

While in response to β-adrenergic stimulation cells of the late stage (day 9 and 10) showed a marked increase in metabolic rate and UCP expression, 5′D-II-activity was stimulated by 0.1 μM isoproterenol only at days 6 to 8. Maximal activation was achieved at day 7 (172 fmol iodide/mg protein per h).

3.7. Changes of receptor equipment during fat cell development

Isoproterenol as an non specific agonist for all known types of β-adrenergic receptors (β1, β2, β3) showed the same effect on 5′D-II activity as its natural equivalent norepinephrine. Both agonists led to an activation of 5′D-II at day 7 by some 600% at a concentration of 0.1 μM after 8 h incubation (174.3 fmol iodide/mg protein per h). When cells were incubated with 0.1 μM of the β3-specific agonist BRL 37344 [16], activation of 5′D-II was lower by some 50% (89.5 fmol iodide/mg protein per h, Fig. 5). A dose-response curve for different agonists revealed that only at a far supra-physiological concentration (1 μM) BRL 37344 led to activation of 5′D-II comparable to the effects of norepinephrine and isoproterenol. In order to find out in how far this was due to a loss of receptor specificity at high concentrations, we tested the β3-agonist/β1-antagonist CGP 12177 [17]. Inhibition of β1-adrenoceptors drastically reduced the effect of β3-mediated stimulation (Fig. 5, inset). Maximal activity was achieved at 10 μM after 8 h treatment with 165.5 fmol iodide/mg protein/h. At this concentration stimulation with BRL 37344 activity of 5′D-II was increased to 300 fmol iodide/mg protein/h, with norepinephrine 216 fmol iodide/mg protein/h, and with isoproterenol 242 fmol iodide/mg protein/h. It seems obvious that the main signal path for 5′D-II activation leads through the β1-adrenergic receptor, which is known to be the main receptor type of immature adipocytes. However, UCP expression is regulated mainly through β3-receptors. This fat cell specific receptor is primarily expressed in mature adipocytes [18,19].

4. Discussion

Brown adipose tissue (BAT) is the main source of non shivering heat production in newborn and small mammals. It plays a pivotal role in the regulation of body temperature under unfavorable climatic conditions [20]. The sympathetic nervous system has been shown to be the main regulatory pathway for activation or deprivation of non shivering thermogenesis in BAT. The expression of the uncoupling protein UCP in brown adipose tissue is under the control of norepinephrine, which acts mainly through the atypical β-adrenergic receptor named β3-adrenoceptor [18,21]. While UCP is a heat producing protein unique to brown fat, the deiodination of thyroxine to the physiologically active triiodothyronine is known for almost all tissues. The type II 5′deiodinase is localized in brown fat, placenta, pituitary, and central nervous system [22]. Its peculiarity is mainly that it generates only T3 and no physiological inactivated rT3 [22]. For brown fat its role is the permission of UCP expression in reaction to chronic cold, i.e. to long-lasting stimulation by norepinephrine [6,23]. The activation of 5′D-II is transient and restricted to a period of about 7 days. Inhibition of 5′D-II by iopanoic acid inhibits the upregulation of UCP expression during cold exposure [7]. In vivo both effects, e.g. activation of 5′D-II and expression of UCP, are simultaneous events. Interestingly, during embryogenesis, the activity of 5′D-II precedes the expression of UCP in brown adipose tissue [24,21]. We tried to evaluate to what extent the activation of 5′D-II might be a permissive step in the maturation of brown adipocytes. Our working hypothesis based on the view that the maturation process of brown adipocytes is a multistep development, functionally comparable to white adipocytes [25]. Therefore we established a primary culture of brown preadipocytes which developed to mature brown adipocytes over a period of 10 days.

Our results show that cultured adipocytes react to an identical β-adrenergic signal differently according to their state of development. In immature brown adipocytes that expressed the lipogenic enzyme LPL but not yet the marker for brown fat UCP in great amounts, the activity of 5′D-II was stimulated to maximal values. However, no metabolic reaction was observed and UCP-mRNA increased rather little,

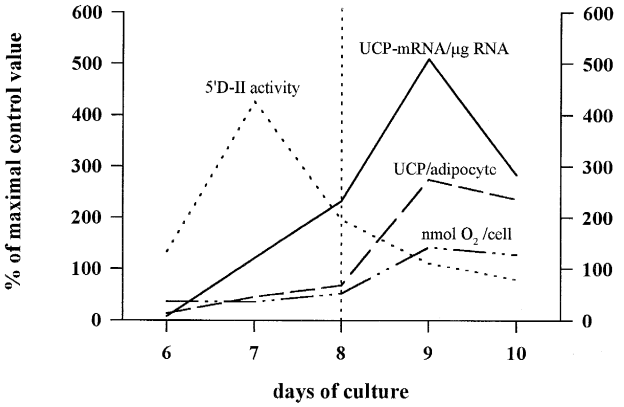

Fig. 6. Summarizing graph of the reaction of brown adipocytes after β-adrenergic stimulation. The period of differentiation is divided into a phase of early differentiation, when metabolism and UCP expression are stimulated only weakly by isoproterenol, but 5′D-II to maximal activity (day 6–8), and a phase of late differentiation, when 5′D-II does not further respond to isoproterenol, but instead metabolism and UCP expression is stimulated to maximal values. The data are taken from Figs. 2, 4 and 5. The maximal control values were set to 100%.

while the corresponding protein was not concerned at all. The opposite affect was observed in mature brown adipocytes that expressed LPL and UCP in considerable amounts. B-Adrenergic stimulation did not effect the activity of 5′D-II. Instead metabolic rate and UCP expression increased to maximal values (Fig. 6).

Measurements of the staining intensity of single adipocytes that were stained for LPL and UCP showed that the LPL content of adipocytes increased steadily during the maturation process, while the content of UCP showed an abrupt increase after the 8th day. This means that the general physiological properties of brown adipocytes change, depending on the state of maturity. We therefore propose to categorize the developmental stages as physiologically discriminable cell types (Fig. 7). There are immature cells with low thermogenic and respiratory capacity. These cells answer to β-adrenergic stimulation with activation of thyroxine (T4) to T3 conversion as one main reaction. This might be a key event in the conversion of these cells into mature brown adipocytes. At the end of maturation cells possess high respiratory and thermogenic capacities and a higher metabolic rate. β-Adrenergic stimulation leads to reactions known from brown adipocytes in vivo: increase in metabolic rate and expression of UCP to maximal values.

The signal pathway changes depending on the state of differentiation. 5′D-II activation is mainly mediated by β1-adrenoceptors characteristic for immature brown adipocytes. UCP expression is mainly controlled by β3-adrenoceptors which are characteristic for mature brown adipocytes. This surely is not true for all species. Pavelka et al. [26] found that the activation of 5′D-II is mediated mainly by β3-adrenoceptors in primary cultures of brown adipocytes of balb/c mice. Interestingly, this activation was also restricted to the 7th day of culture (around conflu-

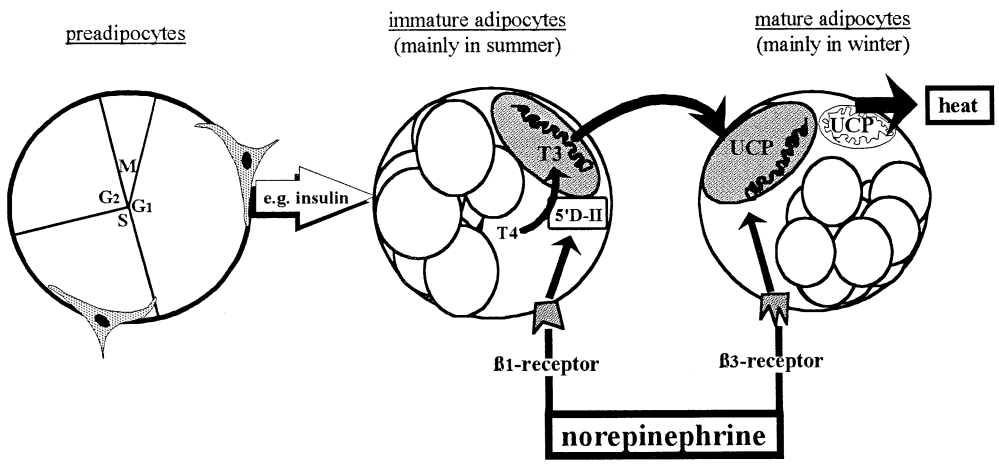

Fig. 7. Hypothetical function of developmental stages of brown adipocytes. Immature adipocytes are looked upon as adipocytes with low thermogenic capacity. They develop to mature brown adipocytes under the influence of long lasting activation of 5′D-II by norepinephrine, which is released to brown adipose tissue in the cold. Conversion of T4 to T3 increases intracellular T3 concentration and allows expression of UCP. This process of modulating cell function might be a decisive step in cold adaptation of the Djungarian hamster in vivo.

ence). This is not necessarily contradictory to our results, since confluence is a prerequisite for cultured adipocytes to undergo differentiation. Furthermore reaction to cold exposure in mice and rats is fundamentally different from that of the Djungarian hamster. While cold exposure in mice and rats leads to a proliferation of brown adipose tissue with a resulting increase in cell number of up to 600% [27,28], in hamsters, total DNA per BAT depot remains unchanged during cold exposure [5]. Thermogenic capacity however, increases to an higher extent in hamsters than in mice.

The adaptive changes in brown adipose tissue of the Djungarian hamster during cold acclimation might base partly on changes in the maturation status of the cell population in the tissue. Brown adipose tissue is known to be a very heterogeneous tissue including various cell types like fibroblasts, epithelial cells, pericytes, and preadipocytes. Only some 30 to 60% of the tissue are adipocytes [29]. From histological investigations it is clear that the population of adipocytes consists of different morphological cell types. These cell types seem to play different roles in the energy homeostasis of the animal [3,24,30,31]. A change in the thermogenic and respiratory capacity of the cell population as a functional entity fits well into results showing that the Djungarian hamster has very much lower thermogenic capacity in summer than in winter [32]. The regulation of the 5′D-II activity in BAT seems to be important for the overall heat production of the animal. High T4 serum levels inhibit its activity in BAT, thus lowering thermogenic capacity of BAT, when the overall heat production is raised by higher metabolic rate in other tissues [33]. Norepinephrine stimulates 5′D-II activity, thus allowing cells to build up higher thermogenic capacity, if the duration of this activation is long enough. Stimulation of 5′D-II activity is probably due to a de novo synthesis of the enzyme, since inhibition of both transcription and translation abolishes this effect [26]. The transient activation of 5′D-II in vivo as well as in vitro, could be a consequence of changing receptor equipment of the cells after increased T4 conversion and the resulting maturation.

Changing physiological function of a given cell population rather than enlarging a cell population with a fixed function could be part of the adaptive concept of the Djungarian hamster. This makes sense especially in the light of its need to reduce body mass in winter, which concerns not only white but also brown fat depots.

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